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**Increased susceptibility of human-PrP transgenic mice to bovine spongiform
encephalopathy following passage in sheep.**

Running title: Sheep BSE transmission to human-PrP transgenic mice

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1 **Abstract**

2 The risk of transmission of ruminant transmissible spongiform encephalopathy (TSE) to
3 humans was thought to be low due to the lack of association between sheep scrapie and
4 incidence of human TSE. However a single TSE agent strain has been shown to cause
5 both bovine spongiform encephalopathy (BSE) and human vCJD, indicating that some
6 ruminant TSEs may be transmissible to humans. While the transmission of cattle BSE to
7 humans in transgenic mouse models has been inefficient, indicating the presence of a
8 significant transmission barrier between cattle and humans, BSE has been transmitted to a
9 number of other species. Here we aimed to further investigate the human transmission
10 barrier following passage of BSE in a sheep. Following inoculation with cattle BSE, gene
11 targeted transgenic mice expressing human PrP showed no clinical or pathological signs
12 of TSE disease. However following inoculation with an isolate of BSE that had been
13 passaged through a sheep, TSE associated vacuolation and proteinase-K resistant PrP
14 deposition were observed in mice homozygous for the codon 129-methionine *PRNP* gene.
15 This observation may be due to higher titres of the BSE agent in sheep, or an increased
16 susceptibility of humans to BSE prions following passage through a sheep. However
17 these data confirm that, contrary to previous predictions, it is possible that a sheep prion
18 may be transmissible to humans and that BSE from other species may be a public health
19 risk.

1 **Introduction.**

2 The transmissible spongiform encephalopathies (TSEs) are a group of fatal infectious
3 neurodegenerative diseases that include scrapie in sheep, bovine spongiform
4 encephalopathy (BSE) in cattle, and Creutzfeldt-Jakob disease (CJD) in humans. TSEs
5 are characterised by the accumulation in the brain of PrP^{Sc}, which is a conformational
6 variant of the normal cellular host prion protein (PrP^C). The abnormal form of the protein
7 is protease resistant, detergent insoluble and aggregates in diffuse or amyloid deposits in
8 the central nervous system (CNS) and lymphoreticular system of infected animals. TSEs
9 are infectious diseases, and can be transmitted between animals of the same and different
10 species by a number of routes including oral, environmental, or iatrogenic exposure. The
11 host range is a specific characteristic of each strain, but TSE agents usually transmit more
12 readily within rather than between species. Low transmission rates are often observed
13 upon transmission to a new species, but on further passage in the new species increased
14 transmission rates and shorter incubation times are usually observed. This effect is
15 referred to as the species barrier. The ability of individual TSE agents to cross a species
16 barrier can be examined experimentally by direct inoculation of different species, or
17 modelled in transgenic mice expressing PrP sequences from these species. Modelling
18 species barriers in mice is particularly important when assessing the risks of infection in
19 humans. Such experiments can assess risk posed by different TSE agents, and also the
20 potential for mutation and adaptation of the agent to the new species. These experiments
21 can highlight changes that may result in the emergence of a new agent strain with a much
22 wider or unknown host range.

23

1 One TSE agent which has shown the ability to transmit to several different species is BSE,
2 where infection has been observed in captive and domestic feline species, and exotic
3 ungulates (kudu & nyala) probably due to ingestion of contaminated feed (13). In 1996 a
4 new variant form of CJD (vCJD) was reported in humans which presented with unusual
5 pathology and an extremely young age range compared to other human TSEs (30). Strain
6 typing experiments demonstrated that vCJD was caused by the same agent strain as BSE
7 (9, 23), indicating that exposure to contaminated foods may have also resulted in
8 transmission of BSE to humans. Humans had previously been thought to be at low risk
9 from contracting ruminant TSEs, as sheep scrapie has been endemic in many countries
10 for hundreds of years without any related foci of human TSE. However the link between
11 BSE and vCJD proved that ruminant TSEs are a public health risk, and that new ruminant
12 TSEs may potentially be transmissible to humans.

13
14 During the BSE epidemic, sheep were undoubtedly exposed to the BSE agent, however
15 no cases of BSE in sheep have been documented in the field. Sheep can however be
16 infected with BSE via oral, intravenous, and intracerebral routes (17, 18), producing
17 clinical TSE with incubation periods ranging from months to years (depending on the PrP
18 genotype of the sheep), proving that this species is susceptible to infection with the BSE
19 agent. It is possible that low level BSE infection did exist in sheep during the height of
20 the BSE epidemic, however it is unknown whether this could have been masked by co-
21 infection with scrapie. BSE has however been documented in goats (14, 25), providing
22 evidence that infection of small ruminants in the field has occurred. Whether the
23 presence of BSE infection in sheep and goats would represent a risk to humans is

1 currently unclear. No previous association between sheep scrapie and human TSE has
2 been documented. This may be due to incompatibility between sheep and human PrP, or
3 the inability of natural scrapie strains to replicate efficiently in a human host. If the agent
4 strain is ultimately responsible for this lack of transmission rather than a sheep/human
5 barrier, it is possible that BSE in other ruminant species may pose a risk to humans.

6
7 In order to address the transmissibility of cattle and sheep derived BSE to humans we
8 performed inoculations of BSE infected sheep brain into a panel of gene targeted
9 transgenic mice expressing human PrP under the same spatial and temporal controls as
10 wild type PrP (4). Three lines of transgenic mice were used representing the genetic
11 diversity in the human population due to the PrP codon 129 methionine/valine
12 polymorphism; HuMM (40%), HuMV (50%) and HuVV (10%). This polymorphism is
13 known to affect human susceptibility to TSE, and to date all confirmed clinical cases of
14 vCJD have occurred in individuals who are methionine homozygous at PrP codon 129.
15 Although previous experiments showed no disease transmission from cattle BSE in any
16 of these human transgenic mouse lines (4), we show here that experimental sheep BSE
17 produced pathological evidence of disease transmission in ~70% of HuMM transgenic
18 mice, suggesting that sheep BSE may be a greater risk to humans than cattle BSE.

1 **Materials and Methods**

2 *Transgenic Mice*

3 Inbred gene targeted human PrP transgenic mice with the codon-129 methionine/valine
4 polymorphism have been described previously (4). Transgenic lines homozygous for the
5 polymorphism were crossed to produce all three genotypes represented in the UK
6 population (designated HuMM, HuMV, and HuVV, respectively) as previously described
7 (4). Brain tissue from a group of uninoculated HuMM and HuVV mice that had
8 previously been allowed to age to over 700 days were utilised as controls in
9 immunohistochemical analyses. In addition a gene targeted bovine transgenic line
10 expressing bovine PrP with the 6-octapeptide repeat region (Bov6), and wild-type
11 129/Ola mice were used as control lines (4) . The Bov6 gene targeted transgenic line is
12 the same line described as “BovTg” in Bishop et al, 2006 (4).

13

14 *Preparation of Inocula*

15 Brain tissue from the cortex of a female, Cheviot sheep (NPU J2501; ARQ/ARQ)
16 experimentally infected via the oral route with cattle BSE (19) was used to prepare 2
17 separate inocula (inoculum-1 and inoculum-2). The sheep was culled with confirmed
18 clinical and pathological BSE at 596 days post inoculation. Natural scrapie isolates
19 (VRQ/VRQ) from the NPU flock were used as controls. Inocula were prepared from
20 cortex tissue in sterile saline at a concentration of 10% (w/v). The cattle BSE brainstem
21 pool used in comparative experiments was supplied by the Veterinary Laboratories
22 Agency, Weybridge UK (infectivity titre $10^{3.3}$ ID₅₀ units/g tissue, measured in RIII mice;
23 M Simmons & R Lockey, personal communication).

1

2 ***Inoculation of transgenic lines.***

3 Experimental sheep BSE inoculum-1 was used to infect groups of gene targeted
4 transgenic mice expressing human PrP with the codon 129 methionine/valine
5 polymorphism (HuMM, HuVV, HuMV), control 129/Ola mice, and gene targeted Bov6
6 mice.(4) In a later experiment, the same transgenic panel was inoculated with
7 experimental sheep BSE inoculum-2 to confirm data obtained with inoculum-1. Data
8 shown (for comparison) from inoculation of HuMM, HuMV, HuVV and Bov6 transgenic
9 mice with a cattle BSE brainstem pool (provided by the Veterinary Laboratories Agency,
10 Weybridge, UK) was generated and published previously (4). The 129/Ola wild type
11 mice inoculated with cattle BSE described here were inoculated in the same manner with
12 the same BSE brainstem pool as part of this work, but were not included in the original
13 publication (4).

14 All mice were intracerebrally (i.c.) inoculated with 0.02ml of inoculum per mouse into
15 the right mid-hemisphere. Following the inoculation, mice were monitored daily and
16 scored once a week for signs of clinical disease. Mice were culled at a pre-defined
17 clinical endpoint (12), or due to welfare reasons, and brain tissue recovered at post
18 mortem. One half of the brain was fixed in formal saline, further trimmed to expose a
19 number of different regions of the brain (frontal cortex, cortex, hippocampus, thalamus,
20 cerebellum and brain stem) then wax embedded to allow 6µm sections to be cut for use in
21 pathological analysis of the tissue. The second half of each brain and the spleen (where
22 available) were snap frozen in liquid nitrogen for biochemical analysis. Each mouse was
23 genotyped post-mortem to confirm PrP genotype. All mouse experiments were reviewed

1 and approved by the Local Ethical Review Committee, and performed under license from
2 the UK Home Office in accordance with the UK Animals (Scientific Procedures) Act
3 1986.

4 5 ***Analysis of vacuolar pathology***

6 Sections were cut (6µm) from each mouse brain and stained using haematoxylin and
7 eosin (H&E). Nine regions of the grey matter (dorsal medulla, cerebellar cortex, superior
8 colliculus, hypothalamus, thalamus, hippocampus, septum, cerebral cortex, and forebrain
9 cerebral cortex) and three regions of white matter (cerebellar white matter, midbrain
10 white matter and cerebral peduncle) were examined and scored on a scale of 0 (no
11 vacuolation) to 5 (severe vacuolation) for the presence and severity of vacuolation. Mean
12 vacuolation scores for each mouse group in each experiment were calculated and plotted
13 with standard error of mean (SEM) against scoring areas to produce a lesion profile (21).

14 15 ***Immunohistochemical analysis of PrP deposition in brain***

16 To identify PrP deposits in the brain, two methods of immunohistochemical (IHC)
17 analysis were employed. (i) ABC kit (Vectastain); brain sections (6µm) were pre-treated
18 using hydrated autoclaving at 121°C for 15 min and exposure to formic acid (95%) for
19 5min prior to incubation with 0.44µg/ml anti-PrP monoclonal antibody 6H4 (Prionics) at
20 room temperature overnight. Biotinylated secondary anti-mouse antibody (Jackson
21 Immuno Research Laboratories, UK) was added at 2.6µg/ml and incubated at room
22 temperature for 1h. PrP^{Sc} was visualised by a reaction with hydrogen peroxidase-
23 activated diaminobenzidine (DAB). (ii) When levels of PrP^{Sc} detected using the ABC kit

were low or zero, the DAKO Catalysed Signal Amplification kit (CSA II (Vectastain)) was used. IHC was performed using the same principals as in (i), but with an additional streptavidin-biotin-peroxidase amplification step (see manufacturer's information). Sections were pre-treated as above prior to incubation with 0.44µg/ml monoclonal antibody 6H4 at room temperature overnight. Anti-mouse immunoglobulins, supplied with the CSA II kit, were added and incubated for 60min at room temperature. PrP^{Sc} detection by a reaction with hydrogen peroxidase-activated DAB.

Immunohistochemical detection of glial activation

To detect astrocyte activation, brain sections (6µm) were incubated with 1.45µg/ml anti-glial fibrillary acidic protein (GFAP, Dako UK Ltd) antibody at room temperature for 1h. To detect microglia activation, brain sections (6µm) were pre-treated using hydrated microwaving for 10min prior to incubation with 0.05µg/ml anti-Iba1 antibody (Wako Chemicals GmbH) at room temperature for 1h. For both primary antibodies, a biotinylated secondary anti-rabbit antibody (Jackson Immuno Research Laboratories, UK) was added at 2.6µg/ml and incubated at room temperature for 1h. Astrocytes and microglia were visualised by a reaction with hydrogen peroxidase-activated DAB.

Detection of amyloid plaques by thioflavin fluorescence

Sections (6µm) were processed and exposed to 1% thioflavin-S (Sigma, UK) solution as described previously (28). Viewed under a fluorescence microscope amyloid deposits fluoresce bright green.

1 ***Identification of PrP^{Sc} in spleen tissue***

2 Spleen tissues that were available from HuMM transgenic mice inoculated with
3 experimental sheep BSE inoculum-2 were screened by the IDEXX HerdChek assay
4 following the manufacturers' guidelines. Buffer volumes for homogenisation were
5 adjusted to ensure that all homogenates were 30% (w/v) for consistency.

6

7 ***Identification of PrP^{Sc} by immunoblot***

8 Brain tissues from HuMM transgenic mice, Bov6 transgenic mice and wild type 129/Ola
9 mice inoculated with experimental sheep BSE inoculum-2 were prepared for analysis by
10 immunoblot. Samples from the experimental sheep BSE source brain and from isolates of
11 natural scrapie were also prepared as controls. Due to the focus of PrP deposition and the
12 amyloid nature of much of the disease associated PrP in HuMM transgenic mice, a
13 centrifugal concentration extraction procedure (including proteinase K digestion) was
14 performed as described previously (24) to maximise the possibility of identifying PrP-res.
15 Samples were loaded and run on 16% Tris-Glycine acrylamide gels (Novex, Invitrogen)
16 at varying concentrations (0.6mg – 64mg brain equivalent) to allow comparison between
17 lanes, and immunoblotted onto PVDF membrane. To achieve a detectable signal,
18 approximately 64mg brain equivalent was loaded from HuMM brain tissue, compared to
19 0.88mg brain equivalent from experimental sheep BSE infected Bov6 controls.
20 Monoclonal antibodies 6H4 (0.1µg/ml) and 12B2 (0.2µg/ml) were used to detect PrP, and
21 bands were visualised using HRP labelled anti-mouse secondary antibody (Jackson
22 Immuno Research Laboratories, UK) and a chemiluminescence substrate (Roche)

1 **Results.**

2 **BSE strain characteristics are retained following transmission in sheep.**

3 For both experimental sheep BSE inoculum-1 and inoculum-2, 100% transmission rates
4 were observed in 129/Ola and Bov6 control mice. Experimental sheep BSE inoculum-1
5 produced incubation times in 129/Ola and Bov6 mice of 474 ± 22 days and 564 ± 8 days
6 respectively (Table 1), similar to those observed in a previous experiment following
7 inoculation of these lines with a cattle BSE brain pool (4). Inoculum-2, prepared from the
8 same BSE infected sheep, gave incubation times of 403 ± 17 days and 487 ± 3 days in
9 129/Ola and Bov6 mice respectively (Table 1). In each line of mice, the lesion profiles of
10 cattle and sheep BSE were similar indicating no change in the targeting properties of BSE
11 following passage through sheep (Figure 1). Lesion profiles of sheep BSE inoculum-1
12 and inoculum-2 were also similar, although the degree of vacuolation was slightly
13 reduced for inoculum-2. This may represent the shortened incubation times observed in
14 these mice. Although these shortened incubation times may reflect a higher level of agent
15 replication in the tissue sample used to prepare inoculum-2, overall the incubation time
16 ratio and targeting of cattle BSE and sheep BSE in control mice indicates no major
17 change in agent characteristics following passage in sheep.

18

19 **Susceptibility of human PrP transgenic mice to experimental sheep BSE.**

20 Following inoculation with experimental sheep BSE inoculum-1, three human transgenic
21 mice (1 x HuMM, 1 x HuVV, 1 x HuMV) were scored as showing clinical signs of TSE
22 disease at 449, 609 and 707 days post inoculation respectively, but had no confirmatory
23 vacuolar pathology in the brain. All other human transgenic mice showed no clinical

1 signs of TSE disease and no TSE associated vacuolar pathology (Table 1). This agreed
2 with previous data generated following inoculation of these human transgenic mouse
3 lines with cattle BSE (4), indicating the presence of a significant transmission barrier to
4 the BSE agent in humans. However, in order to rule out the presence of subclinical
5 disease, the oldest mice were screened for abnormal PrP deposition by
6 immunohistochemistry (IHC) using the anti-PrP antibody 6H4. Of all animals screened,
7 one HuMM mouse which was culled due to old age (706 days post inoculation) showed a
8 small focus of PrP deposition in the thalamus (Figure 2). No other deposition was
9 observed in this animal, or any of the other animals examined.

10

11 In order to confirm this observation, a second separate sample was taken from the same
12 BSE infected sheep brain to prepare inoculum-2, which was used to infect a repeat panel
13 of human transgenic mice. In this experiment, 4 mice (2 x HuVV and 2 x HuMV) were
14 scored as showing clinical signs of TSE disease (between 494-539 days post inoculation),
15 but showed no confirmatory TSE associated pathology in the CNS (vacuolation or PrP
16 deposition). All other human transgenic mice were negative for clinical signs of TSE
17 disease (Table 1). On pathological examination, all HuVV and HuMV mice were
18 negative for vacuolar pathology and PrP deposition. However, 16/23 HuMM mice
19 contained abnormal PrP deposition in the brain, and 3 of the 16 scored positive for
20 vacuolar pathology (Tables 1 and 2). PrP deposition was present in 50% of HuMM mice
21 culled (due to welfare reasons) between 377 and 589 days post inoculation (n=14), and in
22 all of the remaining HuMM mice culled between 600 - 750 days (n=9). Variable patterns
23 and levels of PrP deposition were observed in HuMM mice (Table 2, Figure 3 and Figure

4). Staining was evident in the cochlear nucleus (when identifiable in the tissue section) as punctate-like deposits, in a pattern similar to targeting of BSE in wild type mice (Figure 3a) (9). Punctate perineuronal and intraneuronal staining (Figure 3b & 3c) was also evident in the midbrain and areas of the thalamus. Most strikingly, thioflavin-S fluorescent PrP amyloid plaques were present in the hippocampus, corpus callosum, dorsal lateral geniculate nucleus (dLGN) and thalamus of 8/16 mice positive for TSE pathology (Figure 4h and Supplemental Information: Figure S1). The 8 mice showing thioflavin-S fluorescence were all over 589 days post inoculation (Table 2), indicating that plaque formation was occurring later in the disease process, and that PrP deposition in animals culled prior to 589 days was not amyloid. The PrP plaques were shown to be bi-lateral in a number of tissues where whole brains, or significant portions of the contralateral half had been analysed (Supplemental Information: Figure S2). Florid plaques (associated with vCJD disease in humans) were also evident in the hippocampus (Figure 3d). The vacuolar pathology observed in 3 of the PrP-positive HuMM mice was extremely limited, with the main focus of vacuolation occurring in the hippocampus and thalamus, closely linked with the presence of PrP amyloid plaques (Figure 4d). No PrP deposition was observed in aged (750 day old) uninoculated HuMM and HuVV mice that were stained with 6H4 to control for possible age related PrP deposition due to the transgene (Figure 4c).

As PrP deposition in HuMM transgenic mice was focused to specific brain areas, PrP^{Sc} had to be concentrated by centrifugal purification (SAF prep) to visualise by immunoblot. PrP^{Sc} was extracted from a HuMM transgenic mouse (with PrP deposition; same animal

as shown in Figure 4b, d, f, h), a Bov6 transgenic mouse and a wild type mouse inoculated with experimental sheep BSE inoculum-2 and analysed by immunoblot alongside PrP^{Sc} extracted from the source BSE inoculated sheep, and four scrapie controls. Blots probed with MAb 6H4 revealed a low level of Proteinase K-resistant PrP (PrP-res) in the HuMM mouse compared to Bov6 and 129/Ola controls (Figure 5a). The HuMM sample represented approximately 1/6th brain equivalent, reflecting the foci of deposition in the original tissue (Figure 4d) and the amyloid nature of much of the deposited PrP, which may not have been resolved in the polyacrylamide gel. PrP-res levels were too low in both the HuMM mouse and the original source BSE infected sheep brain to determine the size of the low molecular weight PrP-res band. However when the gel was re-probed with the N-terminal MAb 12B2, reduced levels of staining were observed in lanes containing the source BSE infected sheep brain, and brain homogenate from the HuMM mouse, 129/Ola mouse and Bov6 mouse infected with experimental sheep BSE (Figure 5b). Such reduced staining with MAb 12B2 is characteristic of BSE infection (27, 31)

Glial Activation in infected HuMM transgenic mice

Brain sections from selected HuMM, HuVV and HuMV transgenic mice inoculated with experimental sheep BSE inoculum-2 were screened for activation of astrocytes (anti GFAP) and microglia (anti-Iba1). Aged uninoculated control HuMM and HuVV mice (~750 days) showed modest GFAP and Iba1 immunoreactivity in isolated cells with slender processes (Fig. 4a,e). PrP amyloid deposits were not seen in these animals (Fig. 4c, g). In contrast, astro- and micro-gliosis were observed in HuMM mice inoculated with

1 sheep BSE inoculum-2 in brain areas with abundant PrP amyloid deposition e.g., the
2 lateral geniculate nucleus (LGN) (Figure 4b, d, f, h). Gliosis was not detected in
3 experimental sheep BSE inoculated HuMV and HuVV mice (data not shown).

4 5 ***Peripheral accumulation of PrP^{Sc} in spleen***

6 The IDEXX HerdChek assay was used to analyse spleen tissue available from 17 of the
7 23 HuMM mice inoculated with experimental sheep BSE inoculum-2. Positive assay
8 readouts were obtained for 10 of the 17 spleens analysed (Table 2). The detection of
9 abnormal PrP in spleen tissue by the IDEXX HerdChek assay did not correlate with PrP
10 deposition observed in the brain of the same animal. Of the 10 IDEXX positive samples,
11 three were from mice in which the brain was scored negative by IHC with anti PrP
12 antibody 6H4 (culled at 422 and 658 days post inoculation). In contrast, the three oldest
13 spleen samples available (623, 687 and 750 days post inoculation) were all negative,
14 despite moderate PrP deposition in the brain (Table 2).

15 16 ***Susceptibility of human transgenic mice to natural sheep scrapie.***

17 Brain homogenate from two VRQ/VRQ sheep with clinically and pathologically
18 confirmed scrapie was used to inoculate the transgenic mouse panel. For both isolates,
19 the presence of clinical and pathological signs of TSE disease were observed only in
20 some control 129/Ola wild type mice with long incubation times (Table 1), as has been
21 observed previously in C57 and RIII mice (8). Clinical signs were recorded in several
22 other mice (2 x Bov6 mice, 8 x HuMV and 1 x HuVV mouse), but none of these animals
23 showed signs of TSE associated pathology. No clinical signs of disease were observed in

1 any remaining transgenic mice, and animals were either culled for intercurrent disease or
2 due to old age. No evidence of TSE associated vacuolar pathology or PrP deposition was
3 observed in the human or bovine PrP gene targeted transgenic lines following
4 pathological examination of brain tissue (data not shown).
5

Discussion

While transmission of BSE from cattle to humans via oral exposure has been proposed as the origin of vCJD, the risk posed to humans from BSE infection in other species is currently unknown. Sheep can be experimentally infected with BSE, and it has long been a concern that sheep may have become infected during the BSE epidemic. Although no evidence of such infection has been identified in the field, cases of BSE in goats have been reported (14, 25). Here we have shown that inoculation of experimental sheep BSE into gene targeted HuMM transgenic mice resulted in the identification of TSE related pathology (PrP deposition, vacuolation, and gliosis) in ~70% of the animals overall, and 100% of HuMM mice surviving over 600 days. PrP^{Sc} was detected in brain tissue by immunoblot, and in spleen tissue of several mice using the IDEXX HerdChek assay. Some of the oldest HuMM mice (623-750 days post inoculation) that showed PrP deposition in the brain did not have detectable PrP^{Sc} in spleen. This variability is not unusual, as extremely old mice that show PrP^{Sc} in the brain often have no corresponding deposition in the spleen. This is likely due to loss of germinal centres in the spleen caused by aging (7). No evidence of disease transmission was observed in HuMV or HuVV mice, mirroring the prevalence of vCJD disease observed to date in the UK population. Although the presence of disease pathology indicates agent replication and early phase disease, we cannot predict whether these mice would have developed clinical disease if lifespan had been extended, or if this represents a persistent sub-clinical state. Subpassage of brain material from HuMM mice will be performed to confirm agent replication, and assess adaptation and host range of the agent.

1 Our results cast new light on the existing data concerning BSE transmission to humans.
2 Since the identification of the link between BSE and vCJD, many studies have been
3 performed to demonstrate or model the transmission of BSE to humans using *in vitro*
4 conversion techniques or by inoculation of transgenic mice expressing human PrP. In
5 these transmission studies, cattle BSE has shown limited transmissibility to human PrP
6 transgenic mice (~0-30%), and considerable variation in susceptibility exists between
7 different transgenic lines with varying constructs and protein expression levels (1, 3, 4,
8 11). Highest levels of susceptibility to BSE in mice expressing human PrP with codon
9 129-methionine (~30%) were reported by Asante et al (1) in the 2x over-expression Tg35
10 model (Hu-129M), which included identification of both limited clinical disease and
11 subclinical disease. However lower attack rates of approximately 20% have been
12 reported in Tg650 mice, which have a higher expression level of 129-Met human PrP, of
13 around 5-8 fold (3, 26). The gene targeted transgenic mice utilised in our studies, which
14 express wild type levels of human PrP from the endogenous mouse *Prnp* locus,
15 previously showed no incidence of disease following cattle BSE inoculation (4). These
16 observations have lead to the assumption that over-expression of PrP is essential to model
17 human disease susceptibility in mice, and that rodent models with wild type physiological
18 levels of PrP expression do not live long enough to display signs of disease as would be
19 seen in the longer lifespan human species. It is therefore significant that the inoculation of
20 experimental sheep BSE described here has resulted in the identification of TSE related
21 pathology in the gene targeted human PrP transgenic mice. Additionally, previously
22 published data has shown that short incubation times can be achieved in HuMM and
23 HuVV gene targeted mice (5). Our data show clearly that gene targeted transgenic lines

1 are useful in the study of cross species susceptibility, and that such susceptibility depends
2 on the agent/host combination, rather than the lifespan of a mouse. The inclusion of data
3 obtained in both overexpressing and gene targeted transgenic mice may therefore inform
4 more accurately on the assessment of the true zoonotic potential of a particular TSE
5 isolate.

6
7 The reasons for the increased susceptibility of HuMM mice to experimental sheep BSE in
8 respect to cattle BSE are currently unknown, and are the subject of further investigation
9 in our laboratory. One possible explanation is that our BSE infected sheep brain
10 contained a significantly higher titre of BSE than found in the cattle BSE brainstem pool,
11 resulting in the shortened incubation times in control 129/Ola and Bov6 mice with
12 experimental sheep BSE inoculum-2 compared to cattle BSE, and the pathological
13 features observed in HuMM mice. Incubation times in control mice were also shorter
14 with experimental sheep BSE inoculum-2 compared to inoculum-1, although the ratio
15 between 129/Ola and Bov6 mice was similar for each inoculum. Such variation in
16 incubation time on primary passage of experimental sheep BSE is however common, and
17 has been observed in previous experiments (Supplemental Information: Table S1). The
18 observed difference in incubation times between inoculum-1 and inoculum-2 is therefore
19 not unexpected. Previous studies by Gonzalez et al (22) have shown relatively high
20 infectivity titres of sheep passaged BSE in RIII mice, which were equivalent to those
21 obtained in Romney sheep. The infectivity titre of the cattle BSE brainstem pool used in
22 our transmissions was $10^{3.3}$ ID₅₀ units/g in RIII mice (personal communication; R.
23 Lockey & M. Simmons, Veterinary Laboratories Agency, UK). Those reported by

1 Gonzalez et al (22) for sheep passaged BSE were 10^5 ID₅₀ units/g in RIII mice,
2 suggesting higher titres may indeed be attained in sheep brain. However reported
3 infectivity titres for BSE in cattle have been variable (6, 20, 22). We are therefore
4 performing titration analyses of experimental sheep BSE brainstem in Bov6 mice to
5 provide a direct comparison with titration data already available for the cattle BSE
6 brainstem pool.

7
8 An alternative hypothesis is that passage of BSE through a sheep has altered the strain
9 characteristics of the agent, producing a variant with increased virulence and/or host
10 range. This possibility is supported by recent data describing enhanced virulence of
11 experimental sheep BSE in bovine PrP transgenic mice (BoPrP-Tg110) and porcine PrP
12 transgenic mice (PoPrP-Tg001) compared to cattle BSE (15, 16). BoPrP-Tg110 mice and
13 PoPrP-Tg001 mice (which over-express PrP 8x and 4x respectively) produced
14 significantly shorter incubation times following inoculation with an experimental sheep
15 BSE brainstem pool than with cattle BSE isolates. The differences in incubation time
16 observed in BoPrP-Tg110 mice were maintained on subpassage (15), indicating that the
17 original variation was probably not due to infectivity titre discrepancies between the two
18 BSE sources. However full titration of these tissue homogenates in mice would be
19 required to confirm this was indeed the case. In PoPrP-Tg001 mice, incubation times
20 shortened significantly on subpassage, and were maintained on further subpassage,
21 indicating adaptation to the “new” host (16). In the study described here, lesion profiles
22 obtained in control 129/Ola mice and Bov6 transgenic mice were similar for both cattle
23 and experimental sheep BSE. Although we were unable to resolve the size of the PrP-res

1 low molecular weight band in both the experimental sheep BSE brain homogenate and
2 the experimental sheep BSE infected HuMM mouse, both showed reduced staining with
3 MAb 12B2 which is characteristic of BSE infection (27). There were therefore no
4 differences in strain characteristics between experimental sheep BSE and cattle BSE,
5 with the exception of the transmissibility to HuMM mice (which could be due to
6 increased infectivity titre).

7

8 The altered agent properties of sheep BSE observed by Espinosa and colleagues (15, 16)
9 may suggest that passage through a sheep causes BSE to transmit in a manner more
10 similar to natural scrapie than cattle BSE. To investigate this we inoculated our
11 transgenic panel with two isolates of natural sheep scrapie. No disease pathology was
12 observed in any transgenic mice following inoculation with either isolate of natural
13 scrapie. Hence, in the experiments described here, the susceptibility of the HuMM mice
14 to experimental sheep BSE does not appear to be due to a general susceptibility to ovine
15 prions, but is instead linked specifically to the replication of the BSE strain of agent in
16 sheep brain. Although agent strain characteristics of BSE are not altered when assayed in
17 Bov6 or 129/Ola mice following passage in sheep, both samples of experimental sheep
18 BSE did show positive TSE pathology in HuMM transgenic mice, which has not been
19 seen previously with cattle BSE inoculations in these mice. Whether this is simply due to
20 agent infectivity titre or a more subtle change in agent characteristics is the subject of
21 further analysis in our laboratory.

22

1 Although sheep can be experimentally infected with BSE by oral, intravenous or
2 intracerebral exposure (18), no cases of sheep BSE have been reported in the field. The
3 possible increased risk of disease transmission identified in these studies is thus not of
4 major concern to the public at present. Natural BSE infection has however been identified
5 in goats (14, 25), indicating that small ruminants have been exposed to sources of
6 contamination. We cannot rule out the possibility that sheep may have been infected with
7 BSE during the height of the BSE epidemic, as these animals were undoubtedly exposed
8 to similar feed sources (although with different levels of exposure compared to cattle).
9 Such infection may have been limited and/or localised, and resolved very quickly. BSE in
10 small ruminants may however represent an increased risk to humans due the wider
11 distribution of BSE infectivity identified in peripheral sheep tissues (2, 17, 19, 29)
12 compared to BSE in cattle which is mainly restricted to the CNS (10). While TSEs
13 remain in the environment and continue to infect animals (even at low prevalence) there
14 remains the potential for cross-species transmission and the emergence of TSE isolates
15 with altered strain properties, or host ranges. Our data therefore emphasise the need for
16 continued surveillance to identify, monitor and characterise any new emerging TSE
17 agents that are identified in ruminants, and assess the potential risks posed to other
18 species.

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13

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- 7

1 Table 1. Transmission of cattle BSE, experimental sheep BSE and natural scrapie to gene targeted human and bovine transgenic mice

TSE Isolate	Mouse Line									
	129/Ola		Bov6		HuMM		HuVV		HuMV	
	Incubation time*	Number affected [†]	Incubation time*	Number affected [†]	Incubation time*	Number affected [†]	Incubation time*	Number affected [†]	Incubation time*	Number affected [†]
Cattle BSE (brain pool)	447 ±27	8/8	551 ±12 [‡]	22/22 [‡]	>765	0/18 [‡]	>793	0/22 [‡]	>749	0/23 [‡]
Sheep BSE inoculum-1	474 ±22	11/11	564 ±8	17/17	>812	1/20	>812	0/23	>812	0/23
Sheep BSE inoculum-2	403 ±17	23/23	487 ± 3	24/24	>750	16/23	>650	0/23	>708	0/24
Natural scrapie 1	594, 705	2/15	>811	0/21	>685	0/24	>776	0/22	>671	0/23
Natural scrapie 2	510 ±17	15/23	>647	0/24	>730	0/24	>710	0/24	>682	0/24

- 2
- 3 * Days ± SEM, calculated from mice showing both clinical and pathological signs of TSE. >n represents the survival in days of the oldest
- 4 mouse in groups where both clinical and pathological signs of disease were not observed in any animals
- 5 † number of mice showing TSE pathology (vacuolation and-or PrP deposition)/number of mice inoculated
- 6 ‡ data from Bishop et al 2006 (4)
- 7

1 Table 2. Disease profile of HuMM mice inoculated with experimental sheep BSE inoculum-2

Mouse Number	Survival* (days)	Clinical signs	Vacuolar pathology	Thioflavin	PrP ^d Score [†]	Spleen IDEXX [‡]
1	377	-ve	-ve	-	-	-
2	414	-ve	-ve	-	+	+
3	422	-ve	-ve	-	-	+
4	434	-ve	-ve	-	+	-
5	463	-ve	-ve	-	+	-
6	514	-ve	-ve	-	-	n/a
7	568	-ve	-ve	-	-	+
8	568	-ve	-ve	-	-	+
9	568	-ve	-ve	-	+	+
10	583	-ve	-ve	-	-	n/a
11	583	-ve	-ve	-	+	+
12	583	-ve	-ve	-	+	+
13	589	-ve	-ve	-	-	-
14	589	-ve	-ve	+	++	+
15	603	-ve	-ve	-	++	+
16	622	-ve	-ve	-	++	+
17	623	-ve	-ve	+	++	-
18	681	-ve	-ve	+	+++	n/a
19	687	-ve	+ve	+	++	-
20	692	-ve	-ve	+	+++	n/a
21	705	-ve	-ve	+	+++	n/a
22	723	-ve	+ve	+	+++	n/a
23	750	-ve	+ve	+	+++	-

2
3 *Animals died or culled for welfare reasons.

4 † PrP deposition scored as; -, no deposition; +, very low deposition; ++, low deposition;
5 +++, moderate deposition.

6 ‡ an OD reading above the positive threshold value for the IDEXX assay is represented
7 as +.

8 n/a, no spleen tissue available.

1 Figure Legends.

2 **Figure 1.** Pattern of vacuolation observed in brains of 129/Ola wild type mice (a), and
3 Bov6 mice (b) following inoculation with the cattle BSE brainstem pool, and
4 experimental sheep BSE inoculum-1 and inoculum-2. Profile produced from 9 grey
5 matter areas (1, dorsal medulla; 2, cerebellar cortex; 3, superior colliculus; 4,
6 hypothalamus; 5, thalamus; 6, hippocampus; 7, septum; 8, cerebral cortex; 9, forebrain
7 cerebral cortex) and 3 white matter areas (1*, cerebellar white matter; 2*, midbrain white
8 matter; 3*, cerebral peduncle). Average scores taken from a minimum of six mice per
9 group and plotted against brain area \pm SEM.

10

11 **Figure 2.** Limited PrP^{Sc} accumulation in the thalamus of one HuMM mouse, 706 days
12 post inoculation with experimental sheep BSE (inoculum-1). Panel (b) is higher
13 magnification of the boxed area in panel (a). PrP^{Sc} deposition appears to be restricted to
14 the thalamus (b). Images obtained after staining with anti-PrP antibody 6H4 and
15 counterstained with haemotoxylin. Magnification as shown.

16

17 **Figure 3.** Variation in pattern and location of PrP^{Sc} accumulation in the brain of HuMM
18 mice infected with experimental sheep BSE (inoculum-2). Punctate deposition in the
19 cochlear nucleus (a) similar to BSE targeting in wild type mice. Peri-neuronal (b) and
20 intra-neuronal (c) PrP deposition seen in the midbrain and areas of the thalamus. H&E
21 stain of a large mature florid plaque located in the hippocampus (d). Punctate (e) and
22 linear (f) PrP deposition in the thalamic region. Images a-c and e-f obtained after staining
23 with anti-PrP antibody 6H4 and counterstained with haemotoxylin. Magnification as
24 shown.

1

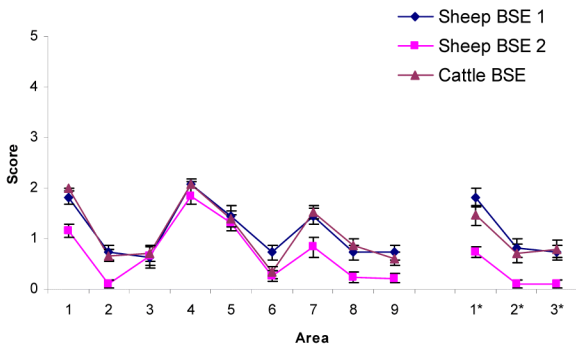
2 **Figure 4.** Comparative analysis of serial sections through the lateral geniculate nucleus
3 (thalamus) of an uninoculated aged (750 days) HuMM mouse and a HuMM mouse
4 infected with experimental sheep BSE (inoculum-2). HuMM mouse infected with sheep
5 BSE (inoculum-2) shows astro- and microgliosis (b & f) visible when stained with anti-
6 GFAP and anti-Iba1 (respectively). Several amyloid plaques are clearly visible
7 fluorescing green with thioflavin-S (h) and stained with anti-PrP antibody 6H4 (d).
8 Sections from a control aged HuMM mouse show mild astro and microgliosis, absence of
9 PrP deposits or amyloid plaques (a, c, e, g). Sections used for immunohistochemical
10 analysis were counterstained with haemotoxylin. x20 Magnification.

11

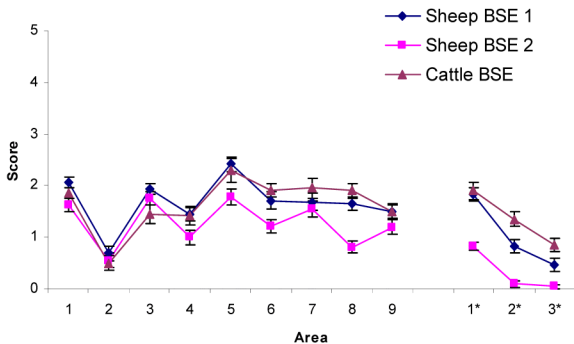
12 **Figure 5.** Comparative western blot (WB) analysis of the proteinase-K resistant
13 fragment (PrP^{Sc}) of the prion protein. Discrimination between BSE and natural scrapie is
14 achieved using two monoclonal antibodies 6H4 (a) and 12B2 (b). Lane 1 – 4.2mg
15 equivalent brain material (mgE) of natural scrapie isolate from the NPU flock. Lane 2 –
16 20mgE of inocula NPU J2501, Cheviot sheep experimentally infected via the oral route
17 with cattle BSE. Lanes 3 & 5 – 1.2 & 1.5mgE of 129/Ola mice infected with a natural
18 scrapie isolate. Lane 4 – 64mgE of HuMM transgenic mouse infected with experimental
19 sheep BSE (inoculum-2). Lanes 6-7 – 2.8 & 0.88mgE of 129/Ola and Bov6
20 (respectively) infected with experimental sheep BSE (inoculum-2). Lane 8 – 0.6mgE
21 ME7/SV control. Molecular markers (M) of the standards are indicated on either side of
22 the panels (kDa).

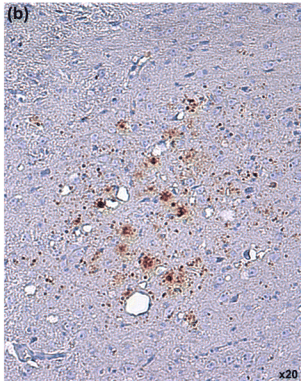
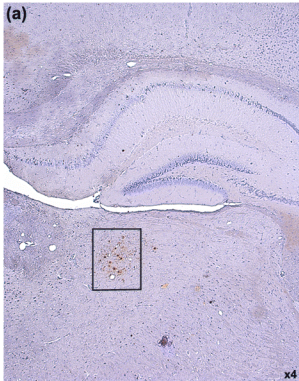
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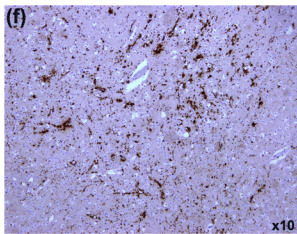
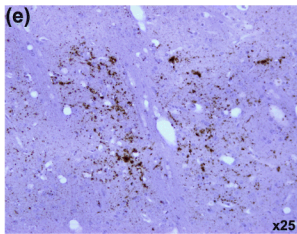
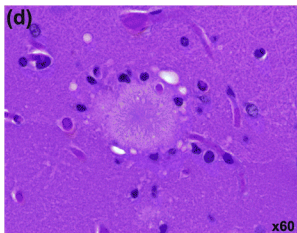
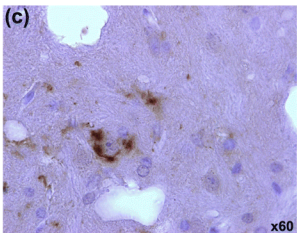
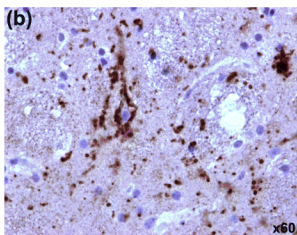
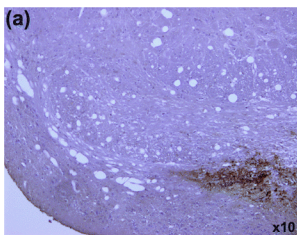
Sheep BSE and cattle BSE in 129/Ola mice



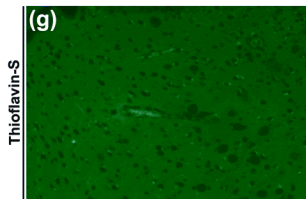
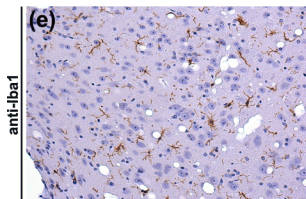
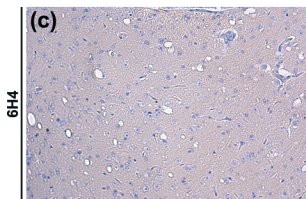
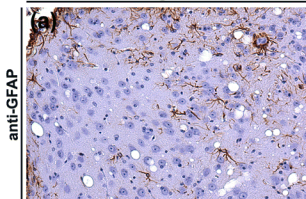
Sheep BSE and cattle BSE in Bov6 mice



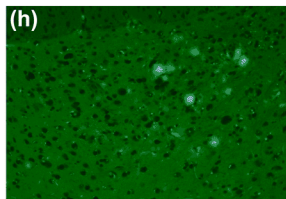
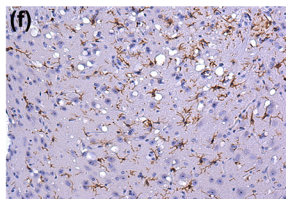
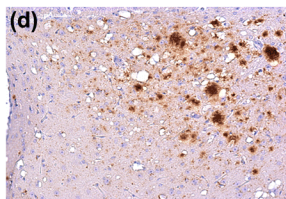
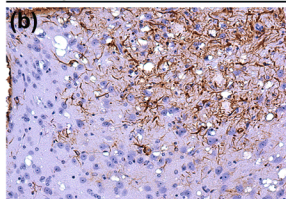


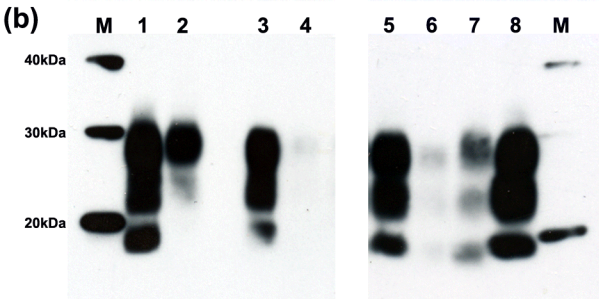
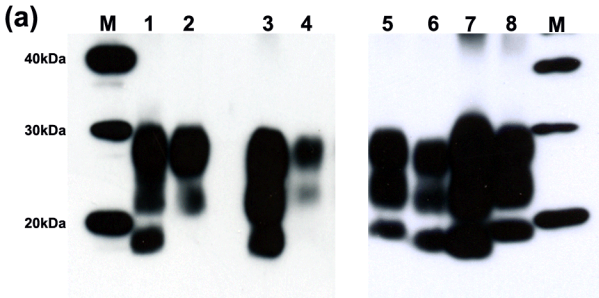


Uninoculated Aged HuMM Mouse

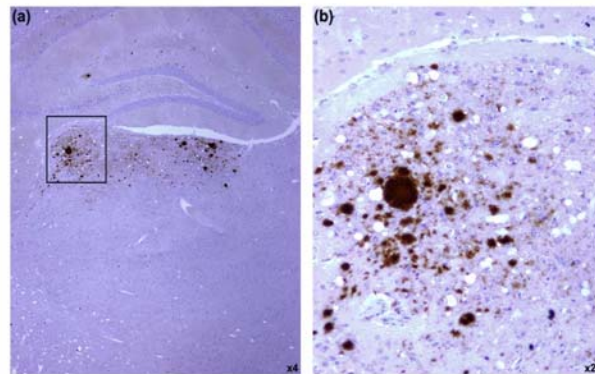


Sheep BSE Infected HuMM Mouse





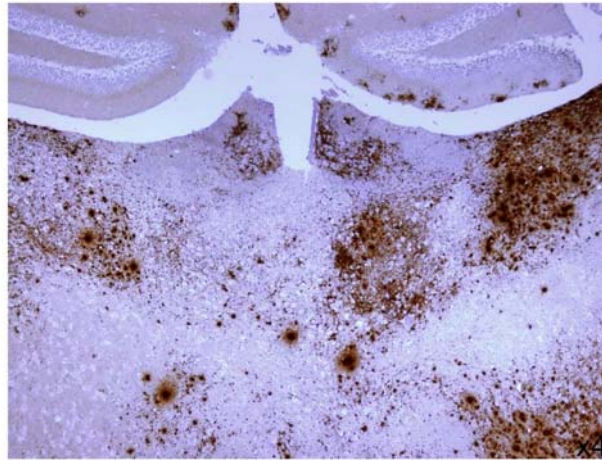
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3 **Figure S1.** PrP^{Sc} deposition in the thalamus of a HuMM mouse inoculated with
4 experimental sheep BSE inoculum-2 (a). Plaques and punctate staining seen in the
5 thalamus and dLGN regions. Highlighted thalamic region (b) with plaques as well as
6 punctate deposition. Stained with anti-PrP antibody 6H4 and counterstained with
7 haemotoxylin. Magnification as shown.

8



1
2 **Figure S2.** Whole brain section of a HuMM mouse inoculated with experimental sheep
3 BSE inoculum-2, illustrating bilateral PrP^{Sc} immunopositive fine punctate, coarse and
4 plaque-like deposits in the thalamus and hippocampus. Stained with anti-PrP antibody
5 6H4 and counterstained with haemotoxylin. x4 magnification.

Sheep	Tissue	Clinical +ve Path +ve (number)	Incubation time range (days)	Incubation time (days \pm SE)	Clinical -ve Path +ve (number)	Survivors, path negative >660dpi (number)
BSE T-1	brain	6	494-607	551 \pm 19	8	4
BSE T-2	brain	7	457-581	527 \pm 18	3	1
BSE T-3	brain	4	511-569	544 \pm 12	6	0
BSE T-4	brain	10	350-499	426 \pm 18	3	0

Table S1. Transmission of experimental sheep BSE to RIII wild type mice.

Brain homogenates prepared from four individual sheep infected with BSE were inoculated separately into groups of RIII mice. Animals were monitored for signs of TSE disease, and culled at a defined clinical endpoint or due to intercurrent illness. The data show the variability that can be observed on primary inoculation of mice with experimental sheep BSE. Incidence of disease ranged from 78% to 100%, with incubation times ranging from 350 days to 607 days. These observations are not uncommon in primary transmission studies.

N. Hunter and M. Bruce. Data from Defra funded project SE1428.